Claims

1. A method for analyzing the C-terminal amino acid sequence of a peptide to be examined, which method comprises the following steps:

a step of preparing a mixture containing a series of reaction products that are obtained from the peptide to be examined by releasing the C-terminal amino acids successively by chemical,

a step of analyzing the differences in molecular weight between said series of reaction products and the original peptide by means of mass spectrometry to measure the decreases in molecular weight associated with the successive release of the C-terminal amino acids, and

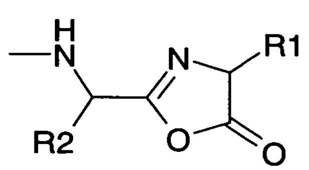
a step of identifying a series of the amino acids removed successively, based on a series of the measured decreases in molecular weight and arranging them from the C-terminus to obtain the information of the C-terminal amino acid sequence of the peptide,

wherein said process for releasing the C-terminal amino acids successively comprises at least the following steps:

a pretreatment step, for providing the protection by means of N-acylation, of allowing an alkanoic acid anhydride and an alkanoic acid both of vapor phase, which are supplied from a mixture of the alkanoic acid anhydride with a small amount of the

alkanoic acid added thereto, to act on a dry sample of said peptide to be examined in a dry atmosphere at a temperature selected in a range of 10ºC to 60ºC and, thereby, applying, to the N-terminal amino group of the peptide as well as to the amino group on the side chain of the lysine residue which may be included in the peptide, N-acylation by the acyl group derived from the alkanoic acid anhydride,

a step of allowing an alkanoic acid anhydride and a perfluoroalkanoic acid both of vapor phase, which are supplied from a mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto, to act on the dry peptide sample after N-acylation protection in a dry atmosphere at a temperature selected in a range of 15ºC to 60ºC and, thereby, releasing the C-terminal amino acids successively in association with a process that at the C-terminus of the peptide, the formation of a 5-oxazolone structure represented by the following general formula (III):



(III)

wherein R1 is a side chain of the C-terminal amino acid of the peptide and R2 is a side chain of the

amino acid residue positioned just before the C-terminal amino acid, is followed by the cleavage of the 5-oxazolone ring, and

a hydrolysis treatment step which comprises applying, to a mixture containing a series of reaction products obtained in said step of releasing the C-terminal amino acids successively, a post-treatment of removing the remaining alkanoic acid anhydride and perfluoroalkanoic acid in a dry state, and then supplying with a basic nitrogen-containing aromatic compound or a tertiary amine compound and water molecules, all of vapor phase, with use of an aqueous solution dissolving the basic nitrogen-containing, aromatic compound or the tertiary amine compound therein, to allow the water molecules to act on the peptides of the reaction products in the presence of the basic nitrogen-containing organic compound to give rise to a hydrolysis treatment, and after that conducting the re-dried up treatment by removing, from the mixture containing a series of reaction products, the remaining basic nitrogen-containing organic compound and water molecules to dry up the mixture,

wherein said step of measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids employs a technique which comprises:

allowing trypsin to act on said mixture, after

the re-dried up treatment, containing a series of the reaction products finished by hydrolysis treatment, in a buffer solution, to carry out the treatment for the enzymatic digestion specific to trypsin of said peptide chain which holds N-acylation protection as for the N-terminal amino group of the peptide chain as well as to the amino group on the side chain of the lysine residue that may be contained in the peptide chain, and thereby, conducting selective cleavage of the C-terminal side peptide bond of each arginine residue that present in the peptide chain to complete peptide fragmentization,

applying a desalting treatment to remove the buffer solution component, followed by recovering and drying the peptide fragments after the digestion treatment by trypsin, followed by drying,

next to that, conducting, as for the dried mixture containing said peptide fragments recovered after the digestion treatment by trypsin, molecular weight measurement for the cationic species as well as molecular weight measurement for anionic species, both of which are generated from the ionization treatment, by means of MALDI-TOF-MS,

with respect to the corresponding ion species, which are measured in said molecular weight measurement for the cationic species as well as molecular weight measurement for anionic species,

judging that the peaks of the peptide fragments each having an arginine residue at the C-terminus, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for cationic species is relatively larger in comparison with the intensity in the molecular weight measurement for anionic species, and judging that the peaks of the C-terminal peptide fragment derived from the original peptide and the C-terminal peptide fragments derived from a series of the reaction products that are obtained by successive release of the C-terminal amino acids, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for anionic species is relatively larger in comparison with the intensity in the molecular weight measurement for cationic species, and

based on a series of the peaks that gives a relatively larger intensity in the molecular weight measurement for anionic species, measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids.

2. A method claimed in Claim 1, wherein a symmetric anhydride of an alkanoic acid having 2 to 4 carbon atoms is used as the alkanoic acid anhydride contained

in the mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto.

3. A method claimed in Claim 2, wherein a symmetric anhydride of a linear chain alkanoic acid having 2 to 4 carbon atoms is used as the symmetric anhydride of said alkanoic acid having 2 to 4 carbon atoms.

4. A method claimed in Claim 1, wherein acetic anhydride is used as the alkanoic acid anhydride contained in said mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto.

5. A method claimed in Claim 1, wherein a perfluoroalkanoic acid of which a pKa is in the range of 0.3 to 2.5 is used as the perfluoroalkanoic acid contained in said mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto.

6. A method claimed in Claim 1, wherein a perfluoroalkanoic acid having 2 to 4 carbon atoms is used as the perfluoroalkanoic acid contained in said mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto.

7. A method claimed in Claim 6, wherein a linear chain perfluoroalkanoic acid having 2 to 4 carbon atoms is used as the perfluoroalkanoic acid having 2 to 4 carbon atoms.

8. A method claimed in Claim 1, wherein a content

of the perfluoroalkanoic acid in the mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto is selected in a range of 1 to 20% by volume relative to the total volume of the alkanoic acid anhydride and the perfluoroalkanoic acid.

9. A method claimed in Claim 1, wherein, in the treatment using said mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto, said dry atmosphere is in a state that not only water but also oxygen have been eliminated.

10. A method claimed in Claim 9, wherein said dry atmosphere is attained by, in an air-tight container, evacuating the inside atmosphere.

11. A method claimed in Claim 1, wherein, in the treatment using said mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto, the temperature used is a temperature selected in a range of 15ºC to 50ºC.

12. A method for analyzing the C-terminal amino acid sequence of a peptide to be examined, which method comprises the following steps:

a step of preparing a mixture containing a series of reaction products that are obtained from the peptide to be examined by releasing the C-terminal amino acids successively by chemical,

a step of analyzing the differences in molecular weight between said series of reaction products and the original peptide by means of mass spectrometry to measure the decreases in molecular weight associated with the successive release of the C-terminal amino acids, and

a step of identifying a series of the amino acids removed successively, based on a series of the measured decreases in molecular weight and arranging them from the C-terminus to obtain the information of the C-terminal amino acid sequence of the peptide,

wherein said process for releasing the C-terminal amino acids successively, as for the sample of the target peptide that has been subjected to separation by gel electrophoresis and is maintained in a state that it is bound on a gel carrier, comprises the following steps:

a step of removing the water solvent impregnated into the gel carrier by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for water, to conduct a dehydration treatment for the gel carrier,

a pretreatment step for the target peptide sample that is still bound on the gel carrier after carrying out said step for dehydration treatment, in which pretreatment step

applying N-acylation protection by the acyl

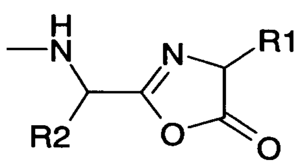
group derived from the alkanoic acid constituting said alkanoic acid anhydride, to the N-terminal amino group of the target peptide with use of a solution of an alkanoic acid anhydride dissolved in a dipolar aprotic solvent that is capable of infiltrating into the gel substance and keeping it in a swollen state is conducted by immersing, at a temperature selected in a range of 30ºC to 80ºC, the gel carrier in the solution of the alkanoic acid anhydride to allow the alkanoic acid anhydride to act on the target peptide sample that is kept in the bound state; and then

removal of said solution is carried out by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for the alkanoic acid anhydride as well as the dipolar aprotic solvent, to conduct termination of the N-acylation reaction and removal of the reaction reagent therefor;

a step of treatment as for the target peptide sample bound on the gel carrier, after the pretreatment step of N-acylation protection, comprising steps of:

immersing, at a temperature selected in a range of 30 ºC to 80 ºC, the gel carrier in a mixed solution of an alkanoic acid anhydride added with a small amount of a perfluoroalkanoic acid in relative ratio thereto dissolved in a dipolar aprotic solvent that is

capable of infiltrating into the gel substance and keeping it in a swollen state, to allow the alkanoic acid anhydride and the perfluoroalkanoic acid to act on the target peptide sample being kept in the bound state; thereby, successive release of the C-terminal amino acids results from the reaction process with use of the mixed solution in which formed is a 5-oxazolone-ring structure represented by the following general formula (III):



(III)

wherein R1 is a side chain of the C-terminal amino acid of the peptide and R2 is a side chain of the amino acid residue positioned just before the C-terminal amino acid, followed by the cleavage of the 5-oxazolone-ring, and

removing the mixed solution used in the reaction for successive release of C-terminal amino acids, by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for the perfluoroalkanoic acid and the alkanoic acid anhydride as well as the dipolar aprotic solvent, to conduct termination of the releasing reaction and removal of the reaction reagents therefor; and

an additional step for hydrolysis treatment and then redehydration treatment, in which step

the hydrolysis treatment for said mixture comprising a series of reaction products obtained by the reaction for successive release of C-terminal amino acids is conducted by immersing the gel carrier in an aqueous solution dissolving a basic nitrogen-containing aromatic compound or a tertiary amine compound therein to allow a water molecule to act, in the presence of said basic nitrogen-containing organic compound, on said peptides of the reaction products being still bound on the gel carrier, and then,

the redehydration treatment for the gel carrier is performed by removing said aqueous solution infiltrated into the gel carrier by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for water; and

wherein said step of measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids employs a technique which comprises:

allowing trypsin being soluble in a buffer solution to act on said mixture, after the re-dried up treatment, containing a series of the reaction products finished by hydrolysis treatment, to carry out the treatment for the enzymatic digestion specific to trypsin of said peptide chain which holds N-

acylation protection as for the N-terminal amino group of the peptide chain as well as to the amino group on the side chain of the lysine residue that may be contained in the peptide chain, and thereby, conducting selective cleavage of the C-terminal side peptide bond of each arginine residue that present in the peptide chain to complete peptide fragmentization,

applying a desalting treatment to remove the buffer solution component, followed by recovering and drying the peptide fragments after the digestion treatment by trypsin, followed by drying,

next to that, conducting, as for the dried mixture containing said peptide fragments recovered after the digestion treatment by trypsin, molecular weight measurement for the cationic species as well as molecular weight measurement for anionic species, both of which are generated from the ionization treatment, by means of MALDI-TOF-MS,

with respect to the corresponding ion species, which are measured in said molecular weight measurement for the cationic species as well as molecular weight measurement for anionic species,

judging that the peaks of the peptide fragments each having an arginine residue at the C-terminus, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight

measurement for cationic species is relatively larger in comparison with the intensity in the molecular weight measurement for anionic species, and judging that the peaks of the C-terminal peptide fragment derived from the original peptide and the C-terminal peptide fragments derived from a series of the reaction products that are obtained by successive release of the C-terminal amino acids, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for anionic species is relatively larger in comparison with the intensity in the molecular weight measurement for cationic species, and

based on a series of the peaks that gives a relatively larger intensity in the molecular weight measurement for anionic species, measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids.

13. A method claimed in Claim 12, wherein a symmetric anhydride of an alkanoic acid having 2 to 4 carbon atoms is used as the alkanoic acid anhydride contained in the mixed solution where a small amount of the perfluoroalkanoic acid in relative ratio to the alkanoic acid anhydride is dissolved.

14. A method claimed in Claim 13, wherein a symmetric anhydride of a linear chain alkanoic acid

having 2 to 4 carbon atoms is used as the symmetric anhydride of said alkanoic acid having 2 to 4 carbon atoms.

15. A method claimed in Claim 12, wherein acetic anhydride is used as the alkanoic acid anhydride contained in the mixed solution where a small amount of the perfluoroalkanoic acid in relative ratio to the alkanoic acid anhydride is dissolved.

16. A method claimed in Claim 12, wherein a perfluoroalkanoic acid of which a pKa is in the range of 0.3 to 2.5 is used as the perfluoroalkanoic acid contained in the mixed solution where a small amount of the perfluoroalkanoic acid in relative ratio to the alkanoic acid anhydride is dissolved.

17. A method claimed in Claim 12, wherein a perfluoroalkanoic acid having 2 to 4 carbon atoms is used as the perfluoroalkanoic acid contained in the mixed solution where a small amount of the perfluoroalkanoic acid in relative ratio to the alkanoic acid anhydride is dissolved.

18. A method claimed in Claim 17, wherein a linear chain perfluoroalkanoic acid having 2 to 4 carbon atoms is used as the perfluoroalkanoic acid having 2 to 4 carbon atoms in the mixed solution where a small amount of the perfluoroalkanoic acid in relative ratio to the alkanoic acid anhydride is dissolved.

19. A method claimed in Claim 12, wherein, in the

mixed solution where a small amount of the perfluoroalkanoic acid in relative ratio to the alkanoic acid anhydride is dissolved, the content ratio of the alkanoic acid anhydride and the perfluoroalkanoic acid is selected in the range of 1 to 20 volumes of the perfluoroalkanoic acid per 100 volumes of the alkanoic acid anhydride.